

APPENDIX A

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Targeting the Kinesin Eg5 to Monitor siRNA Transfection in Mammalian Cells

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ABSTRACT

RNA interference, the inhibition of gene expression by double-stranded RNA, provides a powerful tool for functional studies once the sequence of a gene is known. In most mammalian cells, only short molecules can be used because long ones induce the interferon pathway. With the identification of a proper target sequence, the penetration of the oligonucleotides constitutes the most serious limitation in the application of this technique. Here we show that a small interfering RNA (siRNA) targeting the mRNA of the kinesin Eg5 induces a rapid mitotic arrest and provides a convenient assay for the optimization of siRNA transfection. Thus, dose responses can be established for different transfection techniques, highlighting the great differences in response to transfection techniques of various cell types. We report that the calcium phosphate precipitation technique can be an efficient and cost-effective alternative to Oligofectamine™ in some adherent cells, while electroporation can be efficient for some cells growing in suspension such as hematopoietic cells and some adherent cells. Significantly, the optimal parameters for the electroporation of siRNA differ from those for plasmids, allowing the use of milder conditions that induce less cell toxicity. In summary, a single siRNA leading to an easily assayed phenotype can be used to monitor the transfection of siRNA into any type of proliferating cells of both human and murine origin.

INTRODUCTION

Post-transcriptional gene silencing by double-stranded RNA (RNA interference) is a widespread regulatory pathway among eukaryotes (17). Since its initial observation in *Caenorhabditis elegans*, two primary steps of the un-

derlying mechanism have been identified. Long double-stranded molecules are first processed by an RNase III homolog, DICER, into short oligonucleotides [small interfering RNA (siRNA)] with a duplex region of approximately 19-nucleotide and 2-nucleotide 3' overhangs (1,7). These siRNA are then incorporated into the RNA-induced silencing complex (RISC), creating a sequence-specific nuclease (8). In most vertebrate cells, the presence of double-stranded RNA induces the interferon response pathway, which includes the nonspecific inhibition of translation and RNA degradation (18). By contrast, short double-stranded oligonucleotides of the size generated by DICER are poor inducers, if at all, of the interferon response. Elbashir et al. (5) were the first to demonstrate the feasibility of using siRNA to inhibit gene expression in mammalian cells. Since then, additional reports have confirmed this observation with a growing set of genes, suggesting that this approach will be a major tool for functional genomics in mammalian cells (4,9).

In addition to being almost universal in terms of gene targeting, RNA interference can probably be implemented in a wide variety of cellular contexts. Indeed, although it is still possible that some differentiated cells lack the interfering machinery, there is at this point no documented example of this in mammals. Currently, there appears to be two main aspects to optimize when implementing RNA interference. First, since the RISC recognizes its target through sequence complementarity, it can be expected that the accessibility of the corresponding sequence plays a important role in the efficiency of silencing. Accordingly, the efficiency of silencing can differ significantly between oligoribonucleotides that target different sequences along the same mRNA (10). The second limitation is the penetration of siRNA into cells. Although a direct uptake of oligonucleotides takes place in *Drosophila* S2 cells, a transfection procedure is usually required in mammalian cells. Elbashir and co-workers (5) have reported that for cell lines commonly used for plasmid transfections (e.g., HeLa and NIH 3T3), Oligofectamine™ (Invitrogen SARL,

Cergy Pontoise, France) could be used to introduce siRNA in up to 90% of the cells. However, this protocol cannot be simply transposed to all cell types. Thus, the first difficulty with implementing RNA interference in a new cell type is optimizing the transfection procedure. In particular, it is well known that it is difficult to introduce nucleic acids in hematopoietic cells. In this report we show that an oligonucleotide that targets the Eg5 mRNA provides a powerful tool for optimizing transfection protocols for siRNA.

MATERIALS AND METHODS

Cell Culture

HeLa cells and F9 subclone were routinely maintained in DMEM supplemented with 10% FCS. For monoastrol treatment, the cells were incubated for 16 h in 100 μ M monoastrol (Tocris, Fisher Bioblock Scientific, Illkirch, France).

The K562 cell line was originally established from the pleural effusion of a patient with chronic myeloid leukemia (12), and the UT7 cell line was established from a patient with megakaryoblastic leukemia (11). These cell lines were maintained in α -MEM (Invitrogen SARL) supplemented with 10% FCS and 5 ng/mL recombinant human granulocyte/macrophage-colony stimulating factor (generously provided by Novartis Pharma S.A., Rueil Malmaison, France).

Transfection Procedure

Eg5 oligoribonucleotides were purchased as purified and protected reagents from MWG-Biotech (Courtaboeuf, France). The sequence of the Eg5 sense and antisense strands were 5'-CUGAAGACCUGAAGACAAUdTdT-3' and 5'-AUUGUCUUCAGGUCUUCAGdTdT-3', respectively (centered on position 2263 of GenBank® accession number NM_004523). The sequence of the mutated Eg5 sense and antisense strands were 5'-CACCUCAU-AUCCUUAUCGdTdT-3' and 5'-CG-AUAAGGAAUAUGAGGUGdTdT-3' (centered on position 1065 of GenBank accession number NM_004523; note

that this nucleotide, which is normally a G, is replaced by an A in this mutated sequence). Oligonucleotides were deprotected as recommended by the manufacturer and resuspended in water. Sense and antisense strands were hybridized as previously described (5). The same oligoribonucleotides purchased from other sources, such as Dharmacon (Boulder, CO, USA) and Genset (Paris, France), had similar efficiencies. Moreover, unpurified oligonucleotides were as active as purified ones, while being shipped in larger amounts.

Transfection using Oligofectamine was performed as recommended by the manufacturer. Briefly, 10^5 cells were seeded in 3.5 cm dishes 16 h before transfection. Transfection was performed using 3 μ L Oligofectamine reagent per dish and the indicated amount of siRNA or antisense RNA.

Transfection by calcium phosphate precipitation was performed by a standard procedure (15). Briefly, 10^5 cells were seeded in 3.5 cm dishes 24 h before transfection. The siRNA was diluted with 1 mM Tris, pH 7.8, 0.1 mM EDTA to a final volume of 36.8 μ L. We then added 5.2 μ L 2.5 M CaCl_2 and 42 μ L 2 \times HBS (50 mM HEPES, 280 mM NaCl, 10 mM KCl, and 1.5 mM Na_2HPO_4 , adjusted to pH 7.05 with NaOH), and precipitation was triggered by air bubbling through the solution (injecting twice with the pipetman a volume of air corresponding to its last setting, which is 42 μ L). After 20 min, 84 μ L precipitate were added to one dish. Following an overnight incubation at 37°C, the culture medium was renewed. For RNA preparation, the protocol was scaled up for 10-cm dishes with 5×10^5 cells and 500 μ L precipitate. The silencing efficiency was reproducible and independent from the scale of the experiment.

For electroporation, K562 or UT7 cells were washed twice with serum-free IMDM and resuspended to a final concentration of 10^7 cells/mL in opti-MEM™ (Invitrogen SARL). Subsequently, 0.2 mL cell suspension were mixed with different concentrations of siRNA and electroporated with a Bio-Rad® apparatus. Three different conditions were tested: 300 V and 125 μ F; 280 V and 250 μ F; and 260 V and 960 μ F. The cell viability following electro-

poration was assessed by 7-aminoactinomycin D staining. The 280 V/250 μ F condition was the most efficient for silencing and was used for the dose-response analysis.

Immunofluorescence

Cells were grown and transfected on glass coverslips or, alternatively, treated with monoastrol, and the resulting floating cells were spun on a glass coverslip using a cytopspin. Cells were then fixed by incubation in methanol for 3 min at -20°C. After rehydration in PBS, the cells were incubated with rabbit anti-Eg5 polyclonal antibody or mouse anti- β tubulin monoclonal 2.1 antibody (Sigma-Aldrich, Lyon, France), and then with a Texas Red-coupled goat anti-rabbit or Rho-coupled goat anti-mouse IgG F(ab)₂ fragment (Jackson ImmunoResearch Laboratories, West Grove, PA, USA). The DNA was stained with 4',6 diamino-2-phenylindole (DAPI), 0.25 μ g/mL (Sigma-Aldrich). Coverslips were mounted in Citifluor mounting media (Citifluor Ltd, London, UK). Imaging of immunofluorescence was performed by confocal laser scanning on a Leica TCS-NT/SP (Leica, Heidelberg, Germany), equipped with an air-cooled Argon-Krypton mixed-gas laser and an APOCHROMAT 63 \times 1.32 oil immersion objective.

Western Blot Analysis

Twenty micrograms of protein were separated on a 6.5% SDS-polyacrylamide gel and transferred on a nitrocellulose BAS83 membrane (Schleicher & Schuell, Ecqueville, France). The membrane was incubated with rabbit anti-Eg5 or anti-Kif15 polyclonal antibodies and then with peroxidase-coupled goat anti-rabbit IgG (Jackson ImmunoResearch Laboratories) and revealed with the SuperSignal® West Pico Chemiluminescent Signal kit (Pierce & Warriner, Chester, UK).

Cell Cycle Analysis

The cells were washed in PBS containing 0.5% paraformaldehyde and 0.5% saponin (Sigma Chemical, France) at 4°C for 5 min and then incu-

bated in PBS containing 5 μ g/mL propidium iodide (Sigma-Aldrich) and 100 μ g/mL RNase A at 4°C for 2 h. The samples were then analyzed with a FACSort™ (BD Biosciences, San Jose, CA, USA), and the percentage of cells in the G2/M phase of the cell cycle was calculated using the MultiCycle software (Phoenix Flow Systems, San Diego, CA, USA).

RESULTS AND DISCUSSION

Eg5 is a kinesin-related motor that is involved in the assembly of the mitotic spindle and the migration of chromosomes along the mitotic spindle (16). Inhibition of Eg5 activity, either by microinjection of antibodies (2) or with a specific drug such as monoastrol (14), leads to a monopolar spindle and an arrest of cells in prometaphase. Therefore, inducing a degradation of Eg5 mRNA by RNA interference was expected to lead to a similar phenotype. In this study we used a 19-nucleotide-long duplex RNA with 2-nucleotide 3' overhangs that corresponded to a sequence of the Eg5 coding region that is conserved between man and mouse. In HeLa cells, the introduction of this siRNA with Oligofectamine induced an accumulation of cells in prometaphase and the appearance of monopolar spindles (Figure 1A). This phenotype was only observed with double-stranded oligonucleotides and was comparable with that observed in the presence of monoastrol (Figure 1A). A similar result was reported by Elbashir and co-workers (6,9) with another oligonucleotide targeting the Eg5 mRNA. To confirm that the arrest in prometaphase was due to Eg5 mRNA degradation, Eg5 RNA and protein levels were analyzed on cells transfected 48 h earlier with increasing doses of siRNA. Semiquantitative RT-PCR analysis of Eg5 mRNA revealed a decrease at the lowest dose analyzed, 30 ng per 3-cm dish, reaching 70% at 3 μ g per dish. In parallel experiments, Western blots indicated a 60% decrease in protein levels at the 3- μ g dose. From these bulk analyses, it is not possible to assess precisely the level of suppression with no knowledge of the percentage of cells that have received the oligonucleotide. Together, these results support the in-

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duction of a specific degradation of Eg5 mRNA, leading to an arrest in prometaphase.

While RNA and protein assays are technically demanding and require spe-

cific tools, a blockage in prometaphase can be detected through a variety of experimental approaches, some of which are easily accessible. For adherent cells, the loss of adhesion to the substrate provides the simplest indication of a blockage in prometaphase. Figure

1B illustrates with HeLa cells how this phenotype can be observed by routine inspection under phase contrast microscopy. Moreover, the phenotype was already detectable 24 h after transfection, indicating a rapid blockage of the cell cycle. Because of this rapid re-

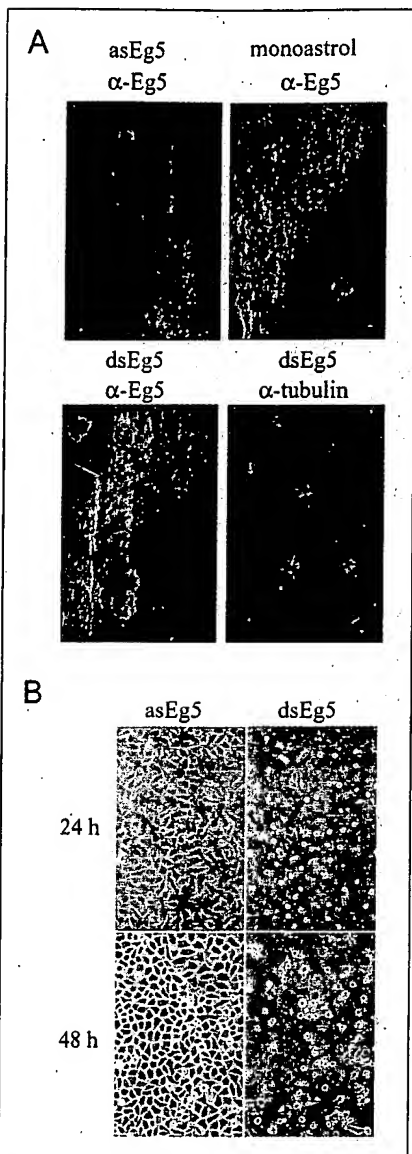


Figure 1. Eg5 siRNA transfection in HeLa cells. (A) Phenotypic analysis of HeLa cells treated with monoastrol, Eg5 antisense RNA (asEg5), or siRNA (dsEg5). Cells were transfected with Oligofectamine or treated with 100 μ M monoastrol for 16 h. Twenty-four hours after transfection or following monoastrol treatment, Eg5, tubulin, and DNA were detected using indicated antibodies (red) and DAPI (blue), respectively. Fluorescence was analyzed by confocal microscopy at 630 \times magnification. (B) Phase contrast microscopy of transfected HeLa cells. The culture was analyzed by phase contrast microscopy at 100 \times magnification 24 h and 48 h after transfection with either antisense (asEg5) or si RNA (dsEg5).

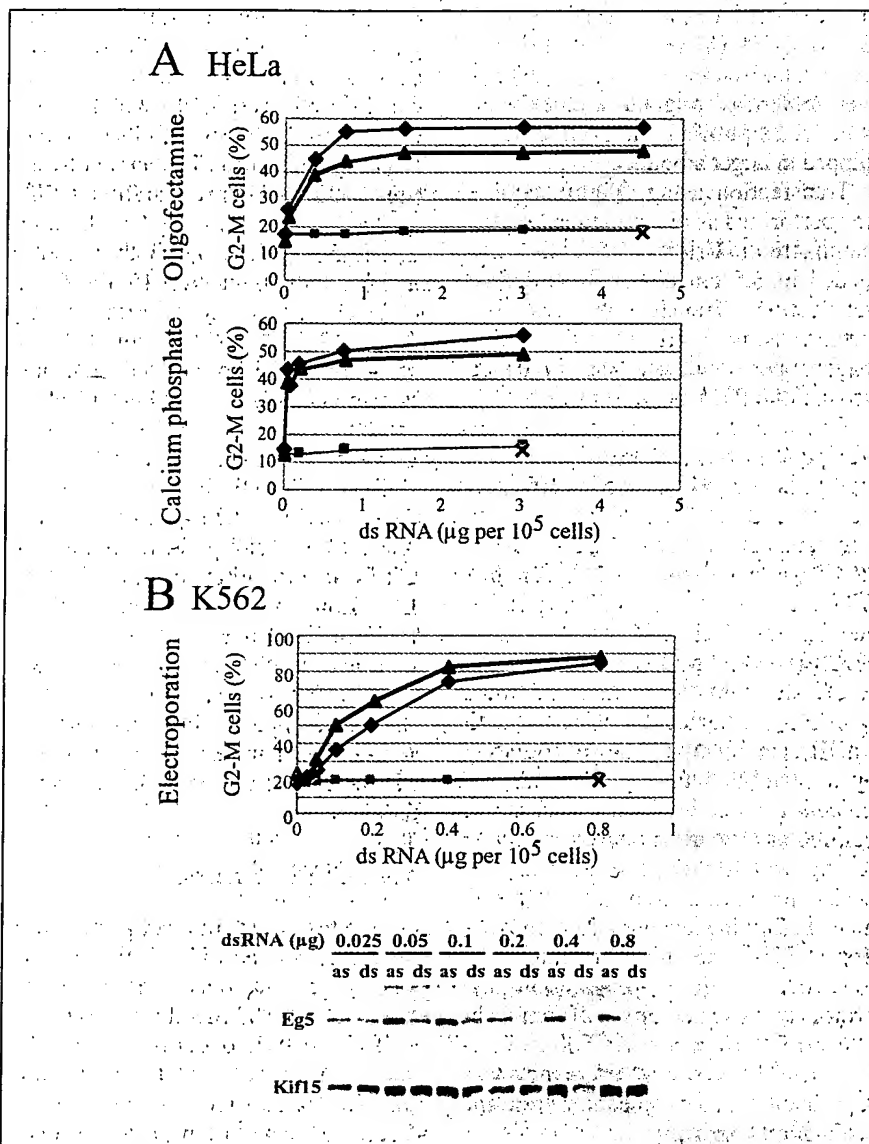


Figure 2. Dose-response analysis of mitotic arrest following Eg5 dsRNA transfection. (A) Dose response in HeLa cells. Cells were transfected with the indicated dose of Eg5 duplex (solid symbols) or antisense RNA (gray squares) using Oligofectamine (upper panel) or calcium phosphate precipitation (lower panel). Two independent experiments are presented with the Eg5 duplex, one with the antisense oligonucleotide. For comparison, the result observed with a mutated Eg5 duplex diverging from the human sequence by one nucleotide is indicated by a cross. The analysis of the cell cycle 48 h after transfection was performed by cytofluorometry after DNA staining with propidium iodide. The percentage of cells in the G2/M phase of the cell cycle was plotted as a function of the RNA dose. (B) Dose response in K562 cells. K562 cells were transfected with the indicated dose of Eg5 duplex (solid symbols) or antisense RNA (gray squares) by electroporation at 280 V and 250 μ F. Two independent experiments are presented with the Eg5 duplex, one with the antisense oligonucleotide, and the result observed with a mutated duplex is indicated by a cross. Cell cycle analysis was performed as in panel A and is presented on the upper panel. An aliquot of the cells transfected by the antisense (as) or duplex (ds) RNA was used to extract the proteins, and Eg5 expression was analyzed by Western blot analysis along with a Kif15 control (lower panel).

sponse, the activity of Eg5 siRNA can also be measured by simply counting the cells 24 h or 48 h after transfection.

Precise quantification of the growth arrest phenotype induced by Eg5 siRNA can be achieved through cytofluorometry. Figure 2A illustrates the use of this approach to measure a dose response of HeLa cells to two different transfection techniques: Oligofectamine and calcium phosphate precipitation. Cytofluorometry was performed on cells stained with propidium iodide 48 h after transfection. Because both transfection protocols entail the creation of inhomogeneous solutions, the doses are expressed in micrograms per 10^5 cells rather than in concentration. Using Eg5 double-stranded oligonucleotides, similar plateaus were reached with both procedures, where 50% to 60% of the cells accumulating in G2/M. For each technique, very reproducible dose responses were observed in independent experiments. Although it has recently been reported that an antisense oligonucleotide could induce gene silencing under some circumstances, the antisense Eg5 oligonucleotide did not exhibit any significant activity in our experiments (13). In addition, a double-stranded oligonucleotide diverging from the human sequence by one nucleotide did not show any activity at the highest dose, which confirms the specificity of the observed phenotype. Importantly, with the calcium phosphate transfection, the plateau was reached with a significantly lower amount of siRNA (100 ng/ 10^5 cells). Calcium phosphate precipitation is a

low-cost transfection procedure that can be performed on large scales that are well adapted to biochemical studies. Our results establish the suitability and the cost effectiveness of this strategy for RNA interference studies. In addition, these results indicate that the penetration of siRNA is more efficient than that of plasmids, as such high transfection efficiency cannot be achieved for these longer molecules even in transient assays.

For cells growing in suspension, calcium phosphate precipitation is inappropriate, while liposomes and cationic lipids give unpredictable results on a new cell line. Electroporation can, a priori, be adapted to all cell types, although high-transfection efficiencies are usually associated with a high cellular toxicity. Reasoning that, similar to calcium phosphate, the requirements for the penetration of siRNA are likely to be different from those of plasmids, we expected that less harsh electroporation conditions could lead to an efficient penetration of siRNA. To explore this possibility, we first used the erythroleukemic cell line K-562, for which efficient conditions of electroporation of long nucleic acid molecules have been reported (19). Using the Eg5 siRNA, we tested electroporation conditions with respect to the arrest in prometaphase and the viability of cells. At 280 V and 250 μ F, the viability of cells following electroporation was higher than 85%. Figure 2B presents a dose response to the Eg5 siRNA. At 0.8 μ g per 10^5 cells, from 85% to 90% of the cells accumulated in G2/M (Figure

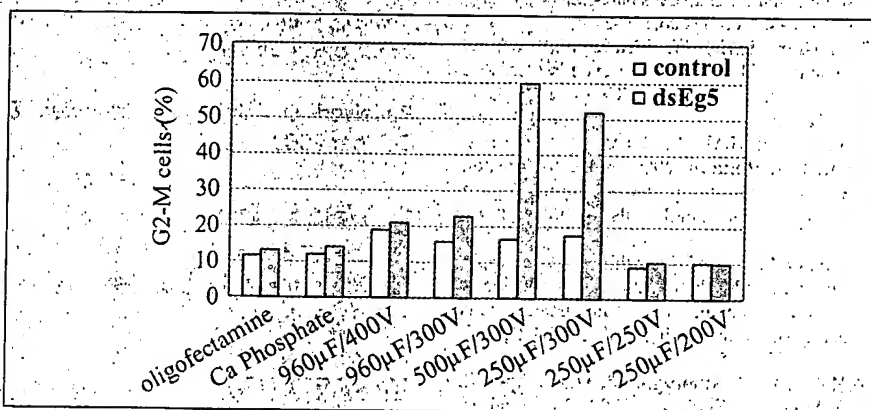
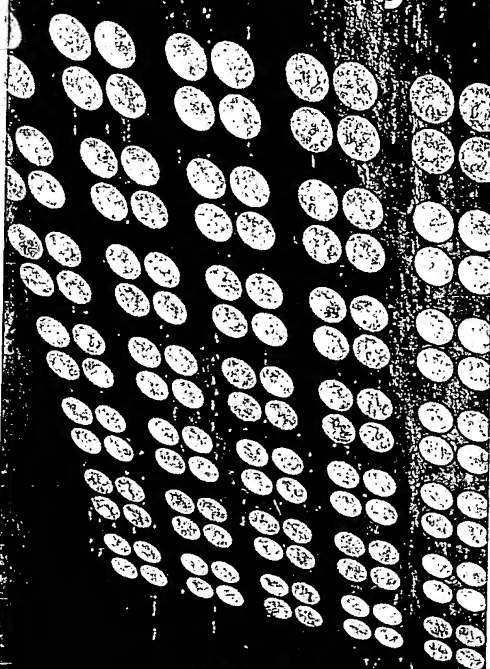


Figure 3. Test experiment on a F9 subclone. The F9 subclone was mock transfected (control) or transfected with the Eg5 duplex RNA using the indicated transfection procedure. Cell cycle analysis was performed as in Figure 2A, 36 h after transfection.

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2B, upper panel). These results indicate that electroporation can enable the penetration of siRNA in a vast majority of the cells. A parallel analysis by Western blot confirmed an important decrease in Eg5 protein (Figure 2B, lower panel). Moreover, the residual expression at the different doses of siRNA correlated with the percentage of cells unaffected in their cell cycle and that presumably had not been transfected. Similar results in terms of both the efficiency of cell cycle arrest and cell viability were obtained with the megakaryocytic cell line UT7 in spite of the low efficiency of plasmid transfection commonly observed in this cell line (data not shown).

Several aspects of Eg5 biology make it an excellent indicator for monitoring RNA interference. First, the endogenous Eg5 gene is expressed in all the proliferating cells that have been analyzed to date. Second, its activity is required for mitosis, and therefore the penetration of siRNA systematically results in an inhibition of growth. This growth inhibition can be evaluated by several approaches that are accessible to all laboratories, from cell counting to DAPI staining and either microscopy or cytofluorometry. Finally, the action of Eg5 siRNA is rapid and best analyzed between 30 h and 48 h. The evaluation of siRNA transfection in a new cell type can therefore be easily monitored with no specific reagents required, provided the targeted cells are proliferating. To illustrate the practical value of Eg5 siRNA, Figure 3 summarizes the testing of different transfection protocols in a subclone of the F9 murine teratocarcinoma cell line. Neither Oligofectamine nor calcium phosphate induced a significant accumulation of cells in G2/M, indicating a low efficiency of transfection. We then explored the feasibility of using electroporation. By varying the parameters of the electrical impulse, conditions could be found that led to an accumulation in G2/M comparable to that observed in HeLa cells (Figure 2A). This result confirmed that, as expected, the same siRNA can be used in murine and human cells. Again, these parameters led to a low cellular toxicity, confirming that the penetration of siRNA does not require transfection protocols as drastic as those for plasmids.

Recently, expression vectors have been designed to enable the synthesis of appropriate siRNA precursors within cells (3). While this can result in a long-term inhibition of the target gene, this strategy entails the transfection of standard plasmids with all the associated limitations. Besides, the use of in vitro synthesized siRNA provides a great flexibility in testing target sequences and for large-scale screenings. Our results illustrate that an efficient transfection of siRNA can be achieved in various cell types, and the Eg5 siRNA provides a versatile tool for the choice and the optimization of the transfection protocol.

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